MANGALORE UNIVERSITY

Jnana Kaveri PG Centre, Chikka Aluvara, Kodagu District, Karnataka

"GREEN SYNTHESIS AND CHARACTERIZATION OF OXIDES OF COPPER AND ZINC NANOPARTICLES FROM ENDEMIC HOLIGARNA SPECIES AND THEIR BIOLOGICAL ACTIVITIES- AN ECO-FRIENDLY APPROACH"

Ph.D. FINAL SYNOPSIS

Submitted to Department of Biochemistry
Mangalore University

FOR THE AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN
BIOCHEMISTRY

By

Mr. Vinayak Lokapur

MU/EXB/Ph.D./CR.117/Bio-chem./2020-2021/E.25

Under the Guidance of

Prof. Manjula Shantaram

Department of Studies and Research in Biochemistry

Mangalore University, Jnana Kaveri Post Graduate Centre, Chikka Aluvara, Kodagu

February 2022

S. NO.	CHAPTER	CONTENTS
1.	I.	**Introduction** **Aim and objectives**
2.	II.	**Review of Literature**
3.	III.	**Materials and Methods**
4.	IV.	**Results and Discussion** Preliminary studies and biosynthesis of zinc oxide and copper oxide nanoparticles using leaf extract of *Holigarna nigra* and their biological activities
5.	V.	Preliminary studies and biosynthesis of zinc oxide and copper oxide nanoparticles using leaf extract of *Holigarna ferrugenia* and their biological activities
6.	VI.	Preliminary studies and biosynthesis of zinc oxide and copper oxide nanoparticles using leaf extract of *Holigarna grahamii* and their biological activities
7.	VII.	**Summary and Conclusion**
8.		**References**
9.		**Publications**

Abbreviations

- **NPs -** Nanoparticles
- **HN -** *Holigarna nigra*
- **HF -** *Holigarna ferrugenia*
- **HG -** *Holigarna grahamii*
- **CuO -** Copper oxide
- **ZnO -** Zinc oxide
- **Hn-ZnONPs -** *Holigarna nigra* - zinc oxide nanoparticles
- **Hf-ZnONPs –** *Holigarna ferrugenia* - zinc oxide nanoparticles
- **Hg-ZnONPs –** *Holigarna grahamii* - zinc oxide nanoparticles
- **Hn-CuONPs -** *Holigarna nigra* - copper oxide nanoparticles
- **Hf-CuONPs -** *Holigarna ferrugenia* - copper oxide nanoparticles
- **Hg-CuONPs -** *Holigarna grahamii* - copper oxide nanoparticles
- **Hrs -** Hours
- **UV-vis spec -** Ultraviolet Visible Spectrophotometer
- **FT-IR -** Fourier Transform Infrared Spectroscopy
- **XRD -** X-Ray Diffraction
- **SEM -** Scanning Electron Microscopy
- **EDAX -** Energy Dispersive X-Ray Analysis
- **HR-TEM -** High Resolution Transmission Electron Microscopy
- **SAED -** Selected Area Electron Diffraction Pattern
- **DLS** - Dynamic Light Scattering
- **FRAP -** Ferric Ion Reducing Antioxidant Power
- **PM -** Phosphomolybdenum
- **DPPH -** 2, 2-Diphenyl-1-picrylhydrazyl
- **HEK 293 -** Human Embryonic Kidney Cells
- **A498 -** Human Renal Carcinoma Cells
- **RCC -** Renal Carcinoma Cells
- **MTT** - (3- (4, 5-Dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide)
- **XTT** - (2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide)
- **FTIC** - Fluorescein-5-isothiocyanate
- **DAPI -** 4’,6-Diamidino-2-phenylindole

CHAPTER I - INTRODUCTION

The nanotechnology is one of the most enthusiastic and emerging fields in modern materials science. According to the International Organization for Standardization (ISO), the prefix nano refers to a size ranging approximately from 1 to 100 nm (1). The use of nanotechnology is far from being new. Norio Taniguchi has first used the term "nanotechnology" in 1974 in reference to machining and finishing dimensional tolerances for semiconductor processes (2). The word "Nano" (Greek word: "dwarf") means extremely small. Nanomaterials can be used in many disciplines of research area, including chemistry, electronics, materials, energy, optics, agriculture, food, and medicine. In the field of medicine there are especially various technology that has developed to cure diseases such as tissue engineering, drug delivery, and biosensors *etc.*, (3). Nanomaterials can be classified according to their source, their dimensions and their constitutive materials as well as the chemical nature of the constituents and divided into organic and inorganic based materials (4). Organic based materials are made of carbon-based nanoparticles, fullerenes, carbon nanotubes, *etc.*, while inorganic based materials include metal oxides such as iron oxide, zinc oxide, copper oxide, titanium dioxide, *etc.*, metals (gold, silver, *etc.*,) and quantum dots like cadmium selenide and cadmium sulfide (5).

Metal-based nanoparticles are called 'a wonder of modern medicine' and have sparked a lot of interest in the field of nanomedicine. Because of their enormous surface area, nanomaterials are well-known for their efficacy in causing minimal side effects and having a targeted effect on cancer cells. Among various metal oxides, zinc oxide (ZnO) and copper oxide (CuO) have been considered in the present work. Zinc oxide is an inorganic compound naturally occurring in crystalline structure. It is a multifunctional semiconducting metal oxide. It is for the most part in yellow to red in colour due to manganese and other impurity (6). Copper oxide (II) or cupric oxide is one of the inorganic compounds with the formula CuO. Both ZnO NPs and CuO NPs replaces the noble metal nanoparticles by its low cost, large surface area, excellent stability, optical, magnetic, catalytic and high conductivity properties (7-10).

According to Selim *et al.*, plant species are a good source for synthesizing nanoparticles on a wide scale since their production rate is quicker than that of other organisms (11). Furthermore, plant-derived nanoparticles contain bioactive components such as alkaloids, polyphenols, flavonoids, terpenoids, and others that aid in the bio-

reduction, production, and stability of nanoparticles as well as varies in different shape and size compared to other organisms such as bacteria, fungi and algae. Traditional folk medicine has been practiced since antiquity and World Health Organization anticipated that almost 80% of the global populations consider traditional systems of medicine as important health sources (12). Many contemporary medicines are isolated from natural sources. Even a toxic plant may be utilized as a possible medicine in treating conditions such as diabetes and cancer when taken at lower concentrations - as Paracelsus, the Father of Toxicology, stated, “The dose makes the poison" (13). Among the various toxic species of plants, Holigarna species are widely known as toxic members of Anacardiaceae family which are endemic to Western Ghats. A total of nine species of Holigarna were reported to occur in India and Myanmar (14, 15). However, The Plant List and Catalogue of Life recognise about 7 accepted species: *Holigarna arnottiana, Holigarna beddomei, Holigarna caustica, Holigarna ferruginea, Holigarna grahamii, Holigarna kurzii,* and *Holigarna nigra*.

These Holigarna species are commonly known as allergic plants which causes ‘Rhus’ - type of contact dermatitis (16). The most commonly known facts about this species are that genus Holigarna have an acrid juice in all its parts (17) and bark in particular, releases white milky latex which will convert to black colour after exposing to air and that is highly irritating to skin (18). Allergic contact dermatitis is a common problem among the outdoor workers of the South Kanara and Konkan area. According to Ayurveda, Holigarna species are mostly allergic to those with pitta prakriti. Holigarna species are the traditional treatment of tribal and local people for haemorrhoids, obesity, skin disorders, and cancer. Despite the claims and usage of Holigarna species in the treatment of many illnesses, genus Holigarna remains unexplored. The only species studied to some extent is *H. anottiana* while other species are untouched (19). Retrospective researches revealed that *H. arnottiana* showed the properties like antioxidant, anti-feedant, anticancer and also allergic potentials (20). The plants considered in the present study are *Holigarna nigra, Holigarna ferrugenia,* and *Holigarna grahamii*. Comprehensive screenings on traditional medicinal plants and their chemical constituents for prevention and treatment of cancer raised considerable interest all over the world (21).

Cancer is a cell aberrant growth capable of uncontrolled proliferating in other healthy bodily tissue, crushing organs, and has become the main cause of human death (22). Kidney cancer is among the top 10 causes of death among various malignancies and is rising at an alarming rate of 3 percent per decade. While renal cell cancer (RCC) is

uncommon, it is projected to account for about 2% of all cancers diagnosed globally in 2018 (23, 24). Surgery is one of the most effective early-stage therapies for kidney cancer, however the survival rate is just one year (25). Whilst other advancements such as radiotherapy and chemotherapy include antimetabolites, alkylating agents and biological agents; however, it is quite difficult to rely on these treatments as they are less effective, costly, long-term diagnosis, risk of damaging other organs and debilitating many adverse effects both for human body as well as to the environment when discarded. It is, therefore, of paramount importance to establish a readily accessible and secure cancer therapy. Over thousand anticancer medicines have been created in the past two decades, but few have been authorized by the Food and Drug Administration, while majority are still in clinical studies (26). Researchers are now focused on developing herbal-based medication with few or no side effects and is both cost efficient, environmentally friendly and a powerful anticancer treatment.

AIM

Aim of the present work was focused on preliminary screening, optimization, green synthesis, and characterization of zinc oxide and copper oxide nanoparticles of selected Holigarna species and to evaluate their antioxidant and anticancer activity.

OBJECTIVES

1. Collection, identification, and preparation of various solvent extracts of *Holigarna nigra, Holigarna ferrugenia,* and *Holigarna grahamii* leaves.
2. Physicochemical analysis and preliminary phytochemical screening of various solvent extracts of *Holigarna nigra, Holigarna ferrugenia,* and *Holigarna grahamii* leaves.
3. Identification of bioactive compounds of different solvent extracts by GCMS analysis and evaluation of their antioxidant activity using *Holigarna nigra, Holigarna ferrugenia*, and *Holigarna grahamii* leaves.
4. Optimization, green synthesis, and characterization of zinc oxide and copper oxide nanoparticles from aqueous leaf extract of *Holigarna nigra*.
5. Optimization, green synthesis, and characterization of zinc oxide and copper oxide nanoparticles from aqueous leaf extract of *Holigarna ferrugenia*.
6. Optimization, green synthesis, and characterization of zinc oxide and copper oxide

nanoparticles from aqueous leaf extract of *Holigarna grahamii*.

7. Evaluation of antioxidant activity of the green synthesized zinc oxide and copper oxide nanoparticles from aqueous leaf extracts of *Holigarna nigra, Holigarna ferrugenia,* and *Holigarna grahamii*.
8. Evaluation of cytotoxicity activity of the green synthesized zinc oxide and copper oxide nanoparticles from aqueous leaf extracts of *Holigarna nigra, Holigarna ferrugenia,* and *Holigarna grahamii*.
9. Evaluation of apoptosis activity of the green synthesized zinc oxide and copper oxide nanoparticles from aqueous leaf extracts of *Holigarna nigra, Holigarna ferrugenia,* and *Holigarna grahamii*.

CHAPTER II - REVIEW OF LITERATURE

This section gives the literature relevant to the present study. This chapter also deals with the information given in the introduction providing the background of green nanotechnology and experimental procedures for the synthesis of ZnO and CuO NPs *via* green routes and the techniques used for their characterizations. The literature survey on an antioxidant and anticancer activity from different plants and their importance in the field of nanotechnology has also been discussed.

CHAPTER III - MATERIALS AND METHODS

This chapter is divided into four sections. Instruments and chemicals used for this research work are given in part I (Reagents and Solvents). In part II, the collection of plants, preparation of extracts, physicochemical and phytochemical screening, GCMS analysis, optimization and synthesis of metal oxide nanoparticles (ZnO NPs and CuO NPs) are discussed. Part III has the demonstration of the instrumental techniques used for characterization of synthesized nanoparticles. The nanomaterials obtained were characterized using the techniques like Ultraviolet Visible Spectrophotometer (UV-vis spec), Fourier Transformed Infrared (FT-IR) Spectroscopy, X-ray Diffraction Analysis (XRD), Scanning Electron Microscope (SEM), Energy Dispersive X-ray Analysis (EDAX), High Resolution of Transmission Electron Microscope (HR-TEM), Selected Area Electron Diffraction (SAED), Dynamic Light Scattering (DLS) and particle size

analyser are given. Part IV deals with the assay procedures of antioxidant and anticancer activities of HN, HF, and HG leaf extracts along with their respective metal oxide nanoparticles (ZnO and CuO NPs). Statistical analysis was carried out wherever necessary. All the values were expressed as mean ± SD (n=3).

COLLECTION AND AUTHENTIFICATION OF PLANT MATERIAL

The plant samples of *H. ferrugeinea* was collected in September, 2020 at the outskirts of Mangalore University (12˚51′19.6″N 75˚01′04.8″E), Dakshina Kannada district, Karnataka whereas *H. grahamii* (12˚24′03.8″N 75˚45′42.9″E) and *H. nigra* (12˚24′03.2″N 75˚45′42.3″E) were collected in September, 2020 from a natural ecosystem at Madikeri road, Kodagu district of Karnataka, India. The plant materials were identified and authenticated by Dr. H. S. Shenoy, Principal Scientist, Pilikula Nisarga Dhama Herbarium, Mangalore, Karnataka, India vide letter no. SKPND: CR: 113: Herbarium Collection/19-20. The herbarium is kept at PND Herbarium, Mangalore as *Holigarna ferrugeinea* Marchand (accession no. 377), *Holigarna grahamii* (Wight) Kurz (accession no. 2366) and *Holigarna nigra* Bourd (accession no. 5107) for further reference.

PREPARATION OF PLANT EXTRACTS

Fresh plant leaves of HN, HF, and HG were cleansed in running tap water followed by distilled water and shade dried. The dried leaf samples were crushed into a coarse powder using mixer grinder. The powdered sample was stored in airtight brown bottle at 4 ºC till further use.

EVALUATION OF PHYSICOCHEMICAL CHARACTERISTICS

Physicochemical properties namely pH, moisture, total ash, acid-insoluble ash content, and elemental studies of HN, HF, and HG leaf extracts were determined using standard protocols.

a) Determination of moisture content

The moisture content of HN, HF, and HG leaves were determined by the weight difference method as mentioned in the standard protocol (27).

b) Determination of total ash content

The total ash content of HN, HF, and HG leaves were determined by following the standard protocol (27).

c) Determination of acid-insoluble ash content

The acid-insoluble ash content of HN, HF, and HG leaves were determined by following the standard protocol (27, 28)

d) Determination of pH

The pH of HN, HF, and HG leaves were determined by using a calibrated digital pH meter by following the method described by AOAC (29).

e) Determination of the elemental constituent of HN, HF, and HG extracts

The standard calibration curve method was used to determine the elemental contents of HN, HF, and HG leaf extracts (30, 31).

PHYTOCHEMICAL SCREENING

Qualitative phytochemical analysis

The various crude extracts of HN, HF, and HG leaves were subjected to preliminary phytochemical screening following standard methods in order to find out the presence of following constituents (30, 32-34).

Quantitative analysis

(a) Estimation of total phenolic content

Estimation of Folin-Ciocalteu test was used to assess the total phenolic content of the extracts by following Noreen *et al.*, (2017) with slight modifications (35). Using Gallic acid as the standard.

(b) Estimation of total flavonoid content

Total flavonoid content of various crude extracts of HN, HF, and HG leaves was evaluated using aluminium colorimetric technique with slight modifications using quercetin as the standard (36).

(c) Estimation of total alkaloid

Determination of total alkaloid content in the extracts was determined by following standard protocol (32).

(d) Estimation of total saponin

Determination of total saponin content in the extracts was determined by following standard protocol (37).

GC-MS ANALYSIS OF VARIOUS SOLVENT EXTRACTS OF HN, HF, AND HG LEAF EXTRACTS

GC-MS was performed at the Centre for Analytical Instrumentation Kerala, India. Identification was supported based on the molecular structure, molecular mass, and calculated fragments. Interpretation of GC-MS was conducted using standard mass spectral database of Wiley and National Institute Standard and Technology (NIST) libraries. The relative percentage amount of every component was calculated by comparing its average peak area to the entire area (38).

OPTIMIZATION OF METAL OXIDE NANOPARTICLES

The physicochemical factors affecting metal oxide nanoparticle synthesis was studied by optimizing the biomass concentration, metal ion concentration and temperature for green synthesis of zinc oxide and copper oxide nanoparticles. These parameters are highly influential in order to maintain the shape, size, morphology, stability, and enhance the product yield (39).

SYNTHESIS OF ZnO AND CuO NPs FROM HN, HF, AND HG LEAF EXTRACTS

Metal oxides like zinc and copper oxide nanoparticles were synthesized using zinc nitrate hexahydrate and cupric nitrate trihydrate respectively by following the previously described method (40) with slight modifications.

ANALYTICAL TECHNIQUES

Characterization of biosynthesized ZnO and CuO nanoparticles

- **UV Visible spectroscopy:** UV-vis spectra were recorded using UV-visible spectrophotometer (Shimadzu, UV-1800). It is a very useful analytical technique as it can be used to determine the amount of substance present in a sample. Double distilled water was used as the reference (blank) solution with matched pair of Teflon stoppered quartz cuvettes of path length 1 cm for the entire study.
- **Fourier Transformed Infrared Spectroscopy (FTIR):** FTIR spectra of all synthesized samples were recorded by Shimadzu. The dried sample is mixed well with KBr (1:100 time) then to get pellet form for FTIR analysis.

- **X-ray Diffraction (XRD):** In the present work, crystal structure, phase and crystallite size of the synthesized nanoparticles were determined by XRD analysis. The X-ray diffraction was carried out by Xpert MPD Philips, Holland.
- **Scanning Electron Microscopy (SEM):** In this study, the morphology analyses were carried out using ZEISS scanning electron microscope.
- **Energy dispersive X-ray analysis (EDAX):** EDAX analysis is a standard method for local identification of elements within a sample in a SEM. In this work, EDAX analysis was performed using ZEISS. This provided quantitative elemental compositions for individual nanostructures.
- **Transmission Electron Microscopy (TEM):** TEM is a unique tool for characterizing crystalline structure and microstructure of materials simultaneously by diffraction and imaging techniques. In this work, TEM analysis was performed using Jeol/JEX 2100.
- **Selected Area Electron Diffraction pattern (SAED):** The SAED pattern can give information on the crystallography and crystal orientation of a sample. In this work, SAED analysis was performed using Jeol/JEX 2100.
- **Particle size distribution analysis:** The size distribution or average size of the synthesized nanoparticles was determined by dynamic light scattering (DLS) method. The DLS technique uses light to determine the size of particles in a solution. A laser emits light at a certain frequency, which is then transmitted through the solution. The analysis was performed using Jeol/JEX 2100.
- **Zeta potential:** The synthesised nanoparticles were evaluated to their surface charge in SZ-100, Horiba Scientific.

***IN VITRO* ANTIOXIDANT ACTIVITY**

Antioxidants are capable of stabilizing or deactivating, free radicals before they attack cells and biological targets to cause various diseases (41). Antioxidants are absolutely critical for maintaining optimal cellular and systemic health and well-being. In the current research, solvent extracts of HN, HF, and HG leaves with concentration ranging from 100 µg/mL to 500 µg/mL and aqueous leaf extract of HN, HF, and HG along with their respective metal oxide nanoparticles (ZnO and CuO NPs) in different concentrations, ranging from 12.5 µg/mL to 200 µg/mL were determined using three different assays:

- 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assay.
- Ferric Ion Reducing Antioxidant Power (FRAP) assay
- Phosphomolybdenum (PM) assay

a) DPPH assay: Free radical scavenging effect of solvent extracts of HN, HF, and HG leaves with concentration ranging from 100 µg/mL to 500 µg/mL and aqueous leaf extract of HN, HF, and HG along with their respective metal oxide nanoparticles (ZnO and CuO NPs) in different concentrations, ranging from 12.5 µg/mL to 200 µg/mL was determined using the DPPH method described by Williams *et al.*, (1995) with slight medications (42).

b) FRAP assay: FRAP assay was used to measure the total antioxidant power of the extracts. The FRAP assay of solvent extracts of HN, HF, and HG leaves with concentration ranging from 100 µg/mL to 500 µg/mL and aqueous leaf extract of HN, HF, and HG along with their respective metal oxide nanoparticles (ZnO and CuO NPs) in different concentrations, ranging from 12.5 µg/mL to 200 µg /mL was performed according to Benzie and Strain (1996) with slight modifications (43).

c) PM assay: The total antioxidant activity of solvent extracts of HN, HF, and HG leaves with concentration ranging from 100 µg/mL to 500 µg/mL and aqueous leaf extract of HN, HF, and HG along with their respective metal oxide nanoparticles (ZnO and CuO NPs) in different concentrations, ranging from 12.5 µg/mL to 200 µg/mL was estimated by PM assay using the standard procedure (44).

***IN VITRO* ANTICANCER ACTIVITY**

(a) Cytotoxicity study

The human renal carcinoma cells (A498) and Human embryonic kidney cells (HEK 293) were procured from the National Centre for Cell Sciences (NCCS), Pune, India. The cytotoxic activity of the aqueous extracts of HN, HF, and HG leaves along with their metal oxide nanoparticles (ZnO and CuO NPs) was evaluated using MTT assay and XTT assay by following Sudha *et al.,* (2020) with slight modifications (45). The cells were treated with a different concentration range of HN, HF, and HG leaves along with their metal oxide nanoparticles (ZnO and CuO NPs), ranging from 6.25 – 200 µg/mL followed by incubation for 48 hrs respectively. Cisplatin was used as the standard drug to compare the effects

induced for HEK293 human embryonic kidney cell and A498 human kidney cancer cell lines.

(b) Selectivity index (SI)

The A498 and HEK293 cells were used to measure the SI. The SI, which represents the cytotoxic selectivity (*i.e.*, drug safety), for both plant extracts and their respective metal oxide nanoparticles was calculated using the formula.

(c) 4, 6-Diamidino-2-phenylindole (DAPI)

DAPI staining was carried out according to the method described by Jordana *et al.,* (2021) with slight modifications (46). Cell nuclear morphology was evaluated by fluorescence microscopy following DAPI staining. The samples were treated with different concentration IC_{25}, IC_{50} and IC_{75} for 24 h.

(d) Annexin V-FITC/PI

Annexin V-FITC (Fluorescein isothiocyanate) was performed to determine cell death mode by following the method of Amin *et al.,* (2017) with slight modifications (47). The samples were treated with different concentration (IC_{25}, IC_{50} and IC_{75}) for 12 h.

STATISTICAL ANALYSIS

The study was carried out in triplicates (n=3) and results were expressed as mean ± standard deviation. Statistical significance between groups was analysed using two-way ANOVA (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) as compared to standard group using GraphPad Prism 5 software (Version 5.03).

CHAPTER IV - RESULTS AND DISCUSSION

Preliminary studies and biosynthesis of zinc oxide and copper oxide nanoparticles using leaf extract of *Holigarna nigra* and their biological activities

PHYSICOCHEMICAL PROPERTIES OF *HOLIGARNA NIGRA*

Table 1: Evaluation of physicochemical characteristics

Tests	HN
Moisture (%)	6.73±0.489
pH	4.70±0.210
Total ash	6.09±0.290
Acid insoluble ash	0.466±0.061

Each value is presented as mean ±SD (n=3)

Table 2: Estimation of micro and macro elements of *Holigarna nigra*

Tests	HN
Sodium (mg/L)	6.826±0.338
Potassium (mg/L)	74.67±3.486
Zinc (mg/L)	0.074±0.008
Iron (mg/L)	0.356±0.015
Chromium (mg/L)	BDL
Lead (mg/L)	BDL
Nickel (mg/L)	BDL

Each value is presented as mean±SD (n=3); BDL - Below detectable level

PRELIMINARY PHYTOCHEMICAL ANALYSIS OF SOLVENT EXTRACTS OF HN LEAVES

Qualitative analysis of crude extracts of HN leaves: The assessment of different qualitative phytochemical screening tests using solvent extracts of HN leaves has revealed the presence of major classes of secondary metabolites such as tannins, saponins, flavonoids, alkaloids, glycosides, cardiac glycosides, terpenoids, steroids, resins, and phenols in all solvents, however, glycosides, cardiac glycosides and terpenoids were absent

in hexane and ethyl acetate extract in common and resins were absent in ethyl acetate, methanol and aqueous. Steroids were present in all solvents except hexane extract.

Quantitative analysis of crude extracts of HN leaves: The highest phenolic content was reported in methanolic extract (0.715±0.068 mg GAE/g) followed by aqueous extract (0.526±0.011), ethyl acetate extract (0.434±0.034 mg GAE/g), and hexane extract (0.016±0.013 mg GAE/g) of *H. nigra*. The highest flavonoid content of *H. nigra* was found in ethyl acetate extract (7.973±14.721 mg QE/g of plant extract) followed by methanol extract (1.946±0.464 mg QE/g), hexane extract (0.480±0.072 mg QE/g) and aqueous extract (0.109±0.038 mg QE/g). The alkaloid and saponin contents were found to be 15.033±0.208 mg/g and saponin 10±2 mg/g respectively.

GC-MS ANALYSIS

Identification of volatile bioactive compounds by GC-MS analysis from various crude extracts of HN leaves.

The presence of phytochemicals from various crude extracts of HN leaves was determined by GC-MS. The bioactive compounds were predicted by their retention time (RT), peak area percentage (%), MW, and molecular formula with the aid of Wiley and NIST libraries. The GC-MS chromatogram of the hexane crude extract revealed five compounds. While, ethyl acetate extract showed the presence of fifteen compounds in the chromatogram. Methanol extract revealed eight active chemicals whereas aqueous extract showed eleven compounds.

ANTIOXIDANT ACTIVITY OF VARIOUS CRUDE EXTRACTS OF HN LEAVES

DPPH assay: Ethyl acetate showed significantly greater DPPH scavenging action at lower concentrations (100 - 200 µg/mL). Hexane, on the other hand, demonstrated a highly significant result as the concentration was increased. All the extracts showed dose dependent inhibition as the concentration was increased.

FRAP assay: Methanol extract showed significantly greater FRAP action at lower concentrations (100 - 400 µg/mL) compared to other extracts. Aqueous extract, on the other

hand, demonstrated a significant result as the concentration was increased. All the extracts showed dose dependent inhibition as the concentration was increased.

PM assay: Ethyl acetate extract showed significantly greater PM action at lower concentrations (100 – 400 µg/ml) compared to other extracts. Methanol extract, on the other hand, demonstrated a significant result as the concentration was increased. All the extracts showed dose dependent inhibition as the concentration was increased.

OPTIMIZATION OF ZINC OXIDE NANOPARTICLES DERIVED FROM AQUEOUS LEAF EXTRACT OF *HOLIGARNA NIGRA* (Hn-ZnO NPs)

Optimization of reaction conditions like the quantity of HN extract, precursor metal ion concentration and temperature were carried out using UV-vis spec.

- **Influence of plant extract concentration:** Hn-ZnO NPs were synthesized by introducing 2.5 mL, 5 mL, and 10 mL of HN plant extract. 5 mL of plant extract revealed a maximum peak at 278 nm.
- **Influence of temperature:** The influence of temperature on the synthesis of Hn-ZnO NPs using HN extract was greatest at 400 °C in a 75 mM concentration
- **Influence of precursor metal ion concentration:** The optimum concentration required by the UV-vis spec at the maximum wavelength of 278 nm was 75 mM, as shown by the spectra, with peak sharpening.

OPTIMIZATION OF COPPER OXIDE NANOPARTICLES DERIVED FROM AQUEOUS LEAF EXTRACT OF *HOLIGARNA NIGRA* (Hn-CuO NPs)

Optimization of reaction conditions like the quantity of HN extract, precursor metal ion concentration and temperature were carried out using UV-vis spec.

- **Influence of plant extract concentration:** Hn-CuO NPs were synthesized by introducing 2.5 mL, 5 mL, and 10 mL of HN plant extract. 5 mL of plant extract revealed a maximum peak at 277 nm.
- **Influence of temperature:** The influence of temperature on the synthesis of Hn-CuO NPs using HN extract was greatest at 600 °C in 100 mM concentration.
- **Influence of precursor metal ion concentration:** The optimum concentration required by the UV-vis spec at the maximum wavelength of 277 nm was 100 mM, as shown by the spectra, with peak sharpening.

SYNTHESIS OF Hn-ZnO AND Hn-CuO NPs

Hn-ZnO NPs were synthesized and characterized using various physicochemical parameters to determine their size, shape, and functionalization after determining the optimal conditions in each of the effective parameters (75 mM of zinc nitrate, 5 mL of HN extract, and calcination at 400 °C temperature). Any alteration in these variables has a direct effect on the biogenesis of Hn-ZnO nanoparticles (40).

Hn-CuO-NPs were synthesized according to the established protocols using the aqueous leaf extract from *H. nigra.* After determining the above optimal conditions in each of the effective parameters, (100 mM of copper nitrate, 5 mL of HN extract and calcination at 600 ºC temperature), the Hn-CuO NPs were synthesized and was characterized using various physicochemical parameters to ascertain their size, shape and functionalization.

CHARACTERIZATION OF Hn-ZnO AND Hn-CuO NPs

UV-vis spectrophotometer: UV-vis spectra of ZnO nanoparticles synthesized by HN shows the absorption peak at 278 nm and revealed the formation of ZnO whereas for CuO nanoparticles, was observed at 277 nm and confirms the formation of CuO.

FTIR analysis: FTIR analysis confirms the functional groups present in the photosynthesized ZnO and CuO nanoparticles. In FTIR spectra of metallic nanoparticles confirmed the presence of alcohols, polyphenols, alkenes, and presence of tertiary alcohol. This shows the special character of biological molecules that can act both as reducing and capping agent in the synthesis of Zinc and Copper oxide nanoparticles.

XRD: XRD showed hexagonal phase wurtzite structure for Hn-ZnO NPs and the average crystallite size of nanoparticles was found to be 12.01 nm. Hn-CuO NPs revealed monoclinic phase with average crystallite size of Hn-CuO nanoparticles was found to be 17.50 nm respectively.

SEM analysis: In Hn-ZnO NPs and Hn-CuO NPs, spherical shape was observed with some agglomerations in the nanoparticles.

EDAX: The quantitative analysis of EDAX spectrum reflects the presence of Zn and O peaks in Hn-ZnO NPs and Cu and O peaks in Hn-CuO NPs, which clearly indicated that the product was of pure ZnO and CuO without impurities. The other weaker signals of K, L and S are owing to use precursor salts for the synthesis of nanoparticles (48).

HR-TEM: The synthesized Hn-ZnO NPs have nanoscale homogeneity of 10-20 nm and are mostly hexagonal forms whereas the synthesized Hn-CuO NPs have nanoscale homogeneity of 17-34 nm and are mostly spherical.

SAED: SAED revealed the ring patterns appeared with the light spots on the dark field. It reflects the crystalline structure of the Hn-ZnO NPs and Hn-CuO NPs.

Zeta potential: Zeta potential provides the zeta value which shows surface the charge and stability. The zeta potential value of Hn-ZnO NPs is -51.4 mV and Hn-CuO NPs is 54.2 mV demonstrating its stability.

Particle size analysis: The average particle size of the Hn-ZnO NPs using DLS method was found to be about 130.1 nm and 127.2 nm for Hn-CuO NPs, both biosynthesized samples exhibited the average particle size fairly higher than the X-ray diffraction method, which might be due to smaller nanoparticles agglomeration.

IN VITRO **ANTIOXIDANT ACTIVITY OF METAL OXIDE NANOPARTICLES AND AQUEOUS HN EXTRACT**

DPPH assay: HN extract showed significantly greater DPPH scavenging action at all concentrations compared to Hn-ZnO NPs, Hn-CuO NPs, and ascorbic acid. Hn-ZnO NPs, on the other hand, demonstrated a highly significant result at lower concentrations (12.5 – 50 µg/mL) compared to ascorbic acid. All extracts showed dose dependent inhibition as the concentration was increased.

FRAP assay: Hn-ZnO NPs and Hn-CuO NPs extract showed significantly greater FRAP action at all the concentrations compared to HN extract and ascorbic acid. Hn-CuO NP extract, on the other hand, demonstrated better scavenging action compared to Hn-ZnO NPs. All extracts showed dose dependent inhibition as the concentration was increased.

PM assay: Metal oxide nanoparticles showed significantly greater PM action at higher concentrations. However, Hn-CuO NP extract demonstrated better PM activity compared to HN extract, Hn-ZnO NPs and ascorbic acid. All extracts showed dose dependent inhibition as the concentration was increased.

IN VITRO ANTICANCER ACTIVITY OF METAL OXIDE NANOPARTICLES AND AQUEOUS HN EXTRACT

Cytotoxicity studies of Hn-ZnO NPs, Hn-CuO NPs and HN extract using MTT assay

MTT assay (HEK293 cell line): At lower concentration (6.25 µg/mL), 90% viable cells were present in HN extract while nearly 80% cells were unharmed in Hn-ZnO NP and Hn-CuO NP extract. However, as the concentration was increased (200 µg/mL), less than 50% living cells can be seen in metal oxide nanoparticles and almost 70% unharmed cells were present in HN extract.

MTT assay (A498 cell line): At lower concentration (6.25 µg/mL), metal oxide nanoparticles inhibited more than 60% cancer cells whereas HN extract inhibited very less amount of cancer cells. As the concentration was increased (200 µg/mL), metal oxide nanoparticles inhibited almost 90% of cancer cells in 48 hrs. These results suggest that the effect of HN extract and metal oxide nanoparticles are dose dependent. Metal oxide nanoparticles are ideal choice at 6.25 µg/mL because no significant effect was observed in normal renal cells at this concentration. Lower the IC_{50} value, higher the anticancer activity. SI values > 2 was considered as high selectivity. Cisplatin was used as the standard drug.

Cytotoxicity studies of Hn-ZnO NPs, Hn-CuO NPs and HN extract using XTT assay

XTT assay (HEK293 cell line): At low concentration (6.25 µg/mL), 90% viable cells were present in HN extract while nearly 80% cells were unharmed in and Hn-ZnO NPs extract Hn-CuO NPs extract. However, as the concentration was increased (200 µg/mL), almost 50% living cells can be seen in metal oxide nanoparticles and 60% unharmed cells were present in HN extract

XTT assay (A498 cell line): At low concentration (6.25 µg/mL), metal oxide nanoparticles inhibited more than 50% cancer cells whereas HN extract inhibited very less amount of cancer cells. As the concentration was increased (200 µg/mL), metal oxide nanoparticles inhibited almost 90% of cancer cells in 48 hrs. These results suggest that the effect of HN extract and metal oxide nanoparticles are dose dependent. Metal oxide nanoparticles are ideal choice at lower concentration because no significant effect was observed in normal

renal cells at this concentration. Lower the IC_{50} value, higher the anticancer activity. SI values > 2 was considered as high selectivity. Cisplatin was used as the standard drug.

APOPTOSIS ACTIVITY OF METAL OXIDE NANOPARTICLES AND AQUEOUS HN EXTRACT

DAPI: DAPI staining revealed that all treated A498 cells visibly appeared to exhibit small nuclei with bright chromatin condensation, blebbing, nuclear fragmentation, apoptotic body formation, chromatin condensation, nuclear shrinkage and nuclear blebbing. The control cells, however, remained intact and evenly shaped. The DAPI staining noticeably showed apoptotic morphological changes in HN extract, Hn-ZnO NPs, and Hn-CuO NPs in terms of both nuclear condensation and cell structure loss.

Annexin V-FITC/PI: The percentage of viable, early apoptotic, late apoptotic, and necrotic cells of negative control (untreated), positive control (Cisplatin), HN extract, Hn-ZnO NPs, Hn- CuO NPs and A498 cells treated for 12 hrs, was determined by a flow cytometer. There was almost 100% of negative control A498 culture cells were viable, and no apoptosis occurred after 12 hrs, of incubation. However, treated cells with HN extract, Hn-ZnO NPs and Hn-CuO NPs, the viable cell percentage reduced significantly at IC_{25}, IC_{50} and IC_{75} respectively. Furthermore, the percentage of cells at apoptotic stage increased significantly at all concentrations compared to control group.

CHAPTER V

Preliminary studies and biosynthesis of zinc oxide and copper oxide nanoparticles using leaf extract of *Holigarna ferrugenia* and their biological activities

PYSICOCHEMICAL PROPERTIES OF *HOLIGARNA FERRUGENIA*

Table 3: Evaluation of physicochemical characteristics

Tests	HF
Moisture (%)	5.566±0.126
pH	4.476±0.251
Total ash	6.433±0.351
Acid insoluble ash	0.513±0.015

Each value is presented as mean ±SD (n=3)

Table 4: Estimation of micro and macro elements of *Holigarna ferrugenia*

Tests	HF
Sodium (mg/L)	6.650±0.595
Potassium (mg/L)	62.706±1.311
Zinc (mg/L)	0.117±0.006
Iron (mg/L)	0.480±0.013
Chromium (mg/L)	BDL
Lead (mg/L)	BDL
Nickel (mg/L)	BDL

Each value is presented as mean±SD (n=3); BDL - Below detectable level

PRELIMINARY PHYTOCHEMICAL ANALYSIS OF SOLVENT EXTRACTS OF HF LEAVES

Qualitative analysis of crude extracts of HF leaves: Phytochemical screening tests using solvent extracts of HF leaves revealed the existence of major classes of secondary metabolites such as tannins, saponins, flavonoids, alkaloids, glycosides, cardiac glycosides, terpenoids, steroids, resins, and phenols in all solvents, however, resins were found to be present only in hexane extract. Glycosides and terpenoids were absent in hexane, ethyl

acetate, and aqueous extracts whereas, cardiac glycosides were found to be absent in hexane extract.

Quantitative analysis of crude extracts of HF leaves: The highest phenolic content was reported in ethyl acetate extract (0.662±0.025 mg GAE/g) followed by methanol extract (0.583±0.018 mg GAE/g), aqueous (0.410±0.014 mg GAE/g) and hexane extract (0.019±0.0008 mg GAE/g) of *H. ferrugenia.* The highest flavonoid content in *H. ferrugenia* was estimated in ethyl acetate extract (9.746±1.339 mg QE/g of plant extract) followed by methanol extract (2.260±0.471 mg QE/g), hexane extract (0.579±0.664 mg QE/g) and aqueous extract (0.125±0.025). The alkaloid and saponin contents were found to be 5.306±0.136 mg/g and 25.466±00.702 mg/g respectively.

GC-MS ANALYSIS

Identification of the volatile bioactive compounds by GC-MS analysis from various crude extracts of HF leaves.

The presence of the phytochemicals in the leaves of *H. ferrugenia* was determined by GC-MS. The bioactive compounds were predicted by their retention time (RT), peak area percentage (%), MW, and molecular formula with the aid of Wiley and NIST libraries. The GC-MS chromatogram of the hexane crude extract revealed thirteen compounds. While, ethyl acetate extract revealed nine compounds. Methanol extract showed the presence of five active chemicals whereas aqueous extract showed seven compounds.

ANTIOXIDANT ACTIVITY OF VARIOUS CRUDE EXTRACTS OF HN LEAVES

DPPH assay: Aqueous extract showed significantly greater DPPH scavenging action at lower concentrations (100 - 200 µg/mL). Hexane, on the other hand, demonstrated a highly significant result as the concentration was increased. All the extracts showed dose dependent inhibition as the concentration was increased.

FRAP assay: Hexane extract showed significantly greater FRAP action at lower concentrations (200 µg/mL) compared to other extracts. Methanol extract, on the other hand, demonstrated a significant result as the concentration was increased. All the extracts showed dose dependent inhibition as the concentration was increased.

PM assay: Hexane extract showed significantly greater PM action at lower concentrations (100 µg/mL) compared to other extracts. Methanol extract, on the other hand, demonstrated a significant result as the concentration was increased. All the extracts showed dose dependent inhibition as the concentration was increased.

OPTIMIZATION OF ZINC OXIDE NANOPARTICLES DERIVED FROM AQUEOUS LEAF EXTRACT OF *HOLIGARNA FERRUGENIA* (Hf-ZnO NPs)

Optimization of reaction conditions like the quantity of HF extract, precursor metal ion concentration and temperature were carried out using UV-vis spec.

- **Influence of plant extract concentration:** Hf-ZnO NPs were synthesized by introducing 2.5 mL, 5 mL, and 10 mL of HF plant extract.10 mL of plant extract revealed a maximum peak at 276 nm.
- **Influence of temperature:** The influence of temperature on the synthesis of Hf-ZnO NPs using HF extract was greatest at 500° C in a 75 mM concentration.
- **Influence of precursor metal ion concentration:** The optimum concentration required by the UV-vis spec at the maximum wavelength of 276 nm was 75 mM, as shown by the spectra, with peak sharpening.

OPTIMIZATION OF COPPER OXIDE NANOPARTICLES DERIVED FROM AQUEOUS LEAF EXTRACT OF *HOLIGARNA FERRUGENIA* (Hf-CuO NPs)

Optimization of reaction conditions like the quantity of HF extract, precursor metal ion concentration and temperature were carried out using UV-vis spec.

- **Influence of plant extract concentration:** Hf-CuO NPs were synthesized by introducing 2.5 mL, 5 mL, and 10 mL of HF plant extract. 10 mL of plant extract revealed a maximum peak at 276 nm.
- **Influence of temperature:** The influence of temperature on the synthesis of Hf-ZnO NPs using HF extract was greatest at 400 °C in a 100 mM concentration.
- **Influence of precursor metal ion concentration:** The optimum concentration required by the UV-vis spec at the maximum wavelength of 276 nm was 100 mM, as shown by the spectra, with peak sharpening.

SYNTHESIS OF Hf-ZnO AND Hf-CuO NPs

The Hf-ZnO NPs were synthesized and characterized using various physicochemical parameters to determine their size, shape, and functionalization after determining the optimal conditions in each of the effective parameters (75 mM of zinc nitrate, 10 mL of HF extract, and calcination at 500 °C temperature). Any alteration in these variables has a direct effect on the biogenesis of Hf-ZnO nanoparticles.

Hf-CuO NPs were synthesized according to the established protocols using the aqueous leaf extract from HF. After determining the above optimal conditions in each of the effective parameters, (100 mM of copper nitrate, 10 mL of HF extract and calcination at 400 ºC temperature), the Hf-CuO NPs were synthesized and was characterized using various physicochemical parameters to ascertain their size, shape and functionalization.

CHARACTERIZATION OF Hf-ZnO AND Hf-CuO NPs

UV-vis spectrophotometer: UV-vis spectra of ZnO nanoparticles synthesized by HN shows the absorption peak at 276 nm and revealed the formation of ZnO whereas for CuO nanoparticles, was observed at 276 nm and confirms the formation of CuO.

FTIR analysis: FTIR analysis confirms the functional groups present in the photosynthesized ZnO and CuO nanoparticles. In FTIR spectra of metallic nanoparticles confirmed the presence of alcohols, polyphenols, alkenes, and presence of tertiary alcohol. This shows the special character of biological molecules that can act both as reducing and capping agent in the synthesis of Zinc and Copper oxide nanoparticles.

XRD: XRD showed hexagonal phase wurtzite structure for Hf-ZnO NPs were exhibited and the average crystallite size of nanoparticles was found to be 8.05 nm. Hf-CuO NPs revealed the monoclinic phase with average crystallite size of Hf-CuO nanoparticles was found to be 9.77 nm respectively.

SEM analysis: In Hf-ZnO NPs and Hf-CuO NPs, spherical shape was observed with some agglomerations in the nanoparticles.

EDAX: The quantitative analysis of EDAX spectrum reflects the presence of Zn and O peaks in Hf-ZnO NPs and Cu and O Peaks in Hf-CuO NPs clearly indicated that the product was of pure ZnO and CuO without impurities. The other weaker signals of K, L and S are owing to use precursor salts for the synthesis of nanoparticles.

HR-TEM: The synthesized Hf-ZnO NPs have nanoscale homogeneity of 12-30 nm and are mostly hexagonal forms whereas the synthesized Hf-CuO NPs have nanoscale homogeneity of 30-51 nm and are mostly spherical.

SAED: SAED revealed the ring patterns appeared with the light spots on the dark field. It reflects the crystalline structure of the Hf-ZnO NPs and Hf-CuO NPs.

Zeta potential: Zeta potential provides the zeta value which shows surface charge and stability. The zeta Potential value of Hf-ZnO NPs is 36.6 mV and Hf-CuO NPs is -32.5 mV demonstrating its stability.

Particle size analysis: The average particle size of the Hf-ZnO NPs using DLS method was found to be about 130.2 nm and 133.7 for Hf-CuO NPs, both biosynthesized samples exhibited the average particle size fairly higher than the X-ray diffraction method, which might be due to smaller nanoparticles agglomeration.

***IN VITRO* ANTIOXIDANT ACTIVITY OF METAL OXIDE NANOPARTICLES AND PLANT EXTRACT**

DPPH assay: HF extract showed significantly greater DPPH scavenging action at all concentrations compared to Hn-ZnO NPs, Hf-CuO NPs, and ascorbic acid. Hn-ZnO NPs, on the other hand, demonstrated a significant result at lower concentrations (12.5 - 50 µg/mL) comparable to ascorbic acid. All extracts showed dose dependent inhibition as the concentration was increased.

FRAP assay: Hf-CuO NPs and Hf-ZnO NPs extract showed significantly greater FRAP action at all the concentrations compared to HF extract and ascorbic acid. Hf-CuO NP extract, on the other hand, demonstrated better scavenging action compared to Hf-ZnO NPs. All extracts showed dose dependent inhibition as the concentration was increased.

PM assay: Metal oxide nanoparticles showed significantly greater PM action at higher concentrations. However, Hf-ZnO NPs extract demonstrated better PM activity compared to HF extract, Hf-CuO NPs and ascorbic acid. All extracts showed dose dependent inhibition as the concentration was increased.

IN VITRO ANTICANCER ACTIVITY OF METAL OXIDE NANOPARTICLES AND AQUEOUS HF EXTRACT

Cytotoxicity studies of Hf-ZnO NPs, Hf-CuO NPs and HF extract using MTT assay

MTT assay (HEK293 cell line): At low concentration (6.25 µg/mL), 90% viable cells were present in HF extract while nearly 80% cells were unharmed in and Hf-ZnO NP and Hf-CuO NPs extract. However, as the concentration was increased (200 µg/mL), less than 40% living cells can be seen in metal oxide nanoparticles and almost 70% unharmed cells were present in HF extract.

MTT assay (A498 cell line): At low concentration (6.25 µg/mL), metal oxide nanoparticles inhibited more than 60% cancer cells whereas HF extract inhibited very less amount of cancer cells. As the concentration was increased (200 µg/mL), metal nanoparticles inhibited almost 90% of cancer cells in 48 hrs. These results suggest that the effect of HF extract and metal oxide nanoparticles are dose dependent. Metal oxide nanoparticles are ideal choice at 6.25 µg/mL because no significant effect was observed in normal renal cells at this concentration. Lower the IC_{50} value, higher the anticancer activity. SI values > 2 was considered as high selectivity. Cisplatin was used as the standard drug.

Cytotoxicity studies of Hf-ZnO NPs, Hf-CuO NPs and HF extract using XTT assay

XTT assay (HEK293 cell line): At low concentration (6.25 µg/mL), 90% viable cells were present in HF extract and Hf-ZnO NPs extract while nearly 70% cells were unharmed in Hf-CuO NPs extract. However, as the concentration was increased (200 µg/mL), less than 50% living cells can be seen in metal nanoparticles and almost 60% unharmed cells were present in HF extract.

XTT assay (A498 cell line): At low concentration (6.25 µg/mL), metal oxide nanoparticles inhibited more than 50% cancer cells whereas HF extract inhibited 30% cancer cells. As the concentration was increased (200 µg/mL), metal oxide nanoparticles inhibited almost 90% of cancer cells in 48 hrs. These results suggest that the effect of HF extract, Hf-ZnO NPs and Hf-CuO NPs extract are dose dependent. Metal oxide nanoparticles are ideal choice at 6.25 µg/mL because no significant effect was observed in normal renal cells at

this concentration. Lower the IC_{50} value, higher the anticancer activity. SI values > 2 was considered as high selectivity. Cisplatin was used as the standard drug.

APOPTOSIS ACTIVITY OF METAL OXIDE NANOPARTICLES AND AQUEOUS HF EXTRACT

DAPI: DAPI staining revealed that all treated A498 cells visibly appeared to exhibit small nuclei with bright chromatin condensation, blebbing, nuclear fragmentation, apoptotic body formation, chromatin condensation, nuclear shrinkage and nuclear blebbing. The control cells, however, remained intact and evenly shaped. The DAPI staining noticeably showed apoptotic morphological changes in HF extract, Hf-ZnO NPs, and Hf-CuO NPs in terms of both nuclear condensation and cell structure loss.

Annexin V-FITC/PI: The percentage of viable, early apoptotic, late apoptotic, and necrotic cells of negative control (untreated), positive control (Cisplatin), HF extract, Hf-ZnO NPs, Hf-CuO NPs and A498 cells treated for 12 hrs, was determined by a flow cytometer. There was almost 100% of negative control A498 culture cells were viable, and no apoptosis occurred after 12 hrs, of incubation. However, treated cells with HF extract, Hf-ZnO NPs and Hf-CuO NPs, the viable cell percentage reduced significantly at IC_{25}, IC_{50} and IC_{75} respectively. Furthermore, the percentage of cells at apoptotic stage increased significantly at all concentrations compared to control group.

CHAPTER VI

Preliminary studies and biosynthesis of zinc oxide and copper oxide nanoparticles using leaf extract of *Holigarna grahamii* and their biological activities

PYSICOCHEMICAL PROPERTIES OF *HOLIGARNA GRAHAMII*

Table 5: Evaluation of physicochemical characteristics

Tests	HG
Moisture (%)	6.79±0.290
pH	4.56±0.383
Total ash	6.75±0.317
Acid insoluble ash	1.47±0.226

Each value is presented as mean ±SD (n=3)

Table 6: Estimation of micro and macro elements of *H. grahamii*

Tests	HG
Sodium (mg/L)	3.45±0.0462
Potassium (mg/L)	59.24±0.2.35
Zinc (mg/L)	0.085±0.005
Iron (mg/L)	0.52±0.046
Chromium (mg/L)	BDL
Lead (mg/L)	BDL
Nickel (mg/L)	BDL

Each value is presented as mean±SD (n=3); BDL - Below detectable level.

PRELIMINARY PHYTOCHEMICAL ANALYSIS OF SOLVENT EXTRACTS OF HG LEAVES

Qualitative analysis of crude extracts of HG leaves: The assessment of different qualitative phytochemical screening tests using solvent extracts of HG leaves has revealed the presence of major classes of secondary metabolites such as tannins, saponins, flavonoids, alkaloids, glycosides, cardiac glycosides, terpenoids, steroids, resins, and phenols in all solvents, however, cardiac glycosides and steroids were absent in hexane

extract and resins were absent in methanol and aqueous. Glycosides and terpenoids were absent in hexane and ethyl acetate extracts.

Quantitative analysis of crude extracts of HG leaves: The highest phenolic content was reported in methanol extract (0.750±0.034 mg GAE/g) followed by ethyl acetate extract (0.626±0.014 mg GAE/g), aqueous (0.233±0.009 mg GAE/g) and hexane extract (0.094±0.935 mg GAE/g) of *H. grahamii*. The highest flavonoid content in *H. grahamii* was estimated in ethyl acetate extract (12.186±1.529 mg QE/g of plant extract) followed by methanol extract (7.006±0.906 mg QE/g), hexane extract (0.770±0.517 mg QE/g) and aqueous (0.105±0.027 mg QE/g). The alkaloid content was found to be 5.8±1.2 mg/g and saponin 3.8±0.6 mg/g.

GC-MS ANALYSIS

Identification of the volatile bioactive compounds by GC-MS analysis of crude extracts of HG leaves.

The presence of the phytochemicals from various crude extracts of *H. grahamii* was determined by GC-MS. The bioactive compounds were predicted by their retention time (RT), peak area percentage (%), MW, and molecular formula with the aid of Wiley and NIST libraries. The GC-MS chromatogram of the hexane crude extract revealed sixteen compounds while ethyl acetate extract revealed thirteen compounds. Methanol extract revealed eight active chemicals whereas aqueous extract showed six compounds.

ANTIOXIDANT ACTIVITY OF VARIOUS CRUDE EXTRACTS OF HG LEAVES

DPPH assay: In comparison to standard ascorbic acid and other solvent extracts, methanol extract showed significantly greater DPPH scavenging action at lower concentrations (100 – 300 µg/mL). Hexane and ethyl acetate, on the other hand, demonstrated a significant result as the concentration was increased. All the extracts showed dose dependent inhibition as the concentration was increased.

FRAP assay: Methanol extract showed significantly greater FRAP action at all concentrations compared to other extracts. All the extracts showed dose dependent inhibition as the concentration was increased.

PM assay: Hexane extract showed significantly greater PM action at lower concentrations (100 µg/mL) compared to other extracts. Methanol extract, on the other hand, demonstrated a significant result as the concentration was increased. All the extracts showed dose dependent inhibition as the concentration was increased.

OPTIMIZATION OF ZINC OXIDE NANOPARTICLES DERIVED FROM AQUEOUS LEAF EXTRACT OF *HOLIGARNA GRAHAMII* (Hg-ZnO NPs)

Optimization of reaction conditions like the quantity of HG extract, precursor metal ion concentration and temperature were carried out using UV-vis spec.

- **Influence of plant extract concentration:** Hg-ZnO NPs were synthesized by introducing 2.5 mL, 5 mL, and 10 mL of HG plant extract. 5ml of plant extract revealed a maximum peak at 276.8 nm.
- **Influence of temperature:** The influence of temperature on the synthesis of Hg-ZnO NPs using HG extract was greatest at 400 °C in a 50 mM concentration.
- **Influence of precursor metal ion concentration:** The optimum concentration required by the UV-vis spec at the maximum wavelength of 276.8 nm was 50 mM, as shown by the spectra, with peak sharpening.

OPTIMIZATION OF COPPER OXIDE NANOPARTICLES DERIVED FROM AQUEOUS LEAF EXTRACT OF *HOLIGARNA GRAHAMII* (Hg-ZnO NPs)

Optimization of reaction conditions like the quantity of HG extract, precursor metal ion concentration and temperature were carried out using UV-vis spec.

- **Influence of plant extract concentration:** Hg-CuO NPs were synthesized by introducing 2.5 mL, 5 mL, and 10 mL of HG plant extract. 10 mL of plant extract revealed a maximum peak at 265 nm.
- **Influence of temperature:** The influence of temperature on the synthesis of Hg-ZnO NPs using HG extract was greatest at 500 °C in a 100 mM concentration.
- **Influence of precursor metal ion concentration:** The optimum concentration required by the UV-vis spec at the maximum wavelength of 265 nm was 100 mM, as shown by the spectra, with peak sharpening.

SYNTHESIS OF Hg-ZnO AND Hg-CuO NPs

The Hg-ZnO NPs were synthesized and characterized using various physicochemical parameters to determine their size, shape, and functionalization after determining the optimal conditions in each of the effective parameters (50 mM of zinc nitrate, 5 mL of HG extract, and calcination at 400 °C temperature). Any alteration in these variables has a direct effect on the biogenesis of Hg-ZnO nanoparticles.

Hg-CuO-NPs were synthesized according to the established protocols using the aqueous leaf extract from HG. After determining the above optimal conditions in each of the effective parameters, (100 mM of copper nitrate, 10 mL of HG extract and calcination at 500 ºC temperature), the Hg-CuO NPs were synthesized and was characterized using various physicochemical parameters to ascertain their size, shape and functionalization.

CHARACTERIZATION OF Hg-ZnO AND Hg-CuO NPs

UV-vis spectrophotometer: UV-vis spectra of ZnO nanoparticles synthesized by HN shows the absorption peak at 276.8 nm and revealed the formation of ZnO whereas for CuO nanoparticles, was observed at 265 nm and confirms the formation of CuO.

FTIR analysis: FTIR analysis confirms the functional groups present in the photosynthesized ZnO and CuO nanoparticles. In FTIR spectra of metallic nanoparticles confirmed the presence of alcohols, polyphenols, alkenes, and presence of tertiary alcohol. This shows the special character of biological molecules that can act both as reducing and capping agent in the synthesis of Zinc and Copper nanoparticles.

XRD: XRD showed hexagonal phase wurtzite structure for Hg-ZnO NPs were exhibited and the average crystallite size of nanoparticles was found to be 9.20 nm. Hg-CuO NPs revealed monoclinic phase with average crystallite size of Hg-CuO nanoparticles was found to be 10.62 nm respectively.

SEM analysis: In Hg-ZnO NPs and Hf-CuO NPs, spherical and hexagonal shape was observed with some agglomerations in the nanoparticles.

EDAX: The quantitative analysis of EDAX spectrum reflects the presence of Zn and O peaks in Hg-ZnO NPs and Cu and O Peaks in Hg-CuO NPs, which clearly indicated that the product was of pure ZnO and CuO without impurities. The other weaker signals of K, L and S are owing to use precursor salts for the synthesis of nanoparticles.

HR-TEM: The synthesized Hg-ZnO NPs have nanoscale homogeneity of 9-15 nm and are mostly hexagonal forms whereas the synthesized Hg-CuO NPs have nanoscale homogeneity of 20-40 nm and are mostly spherical.

SAED: SAED revealed the ring patterns appeared with the light spots on the dark field. It reflects the crystalline structure of the Hg-ZnO NPs and Hg-CuO NPs.

Zeta potential: Zeta potential provides the zeta value which shows surface charge and stability. The zeta potential value of Hg-ZnO NPs is -26.7 mV and Hg-CuO NPs is -28.3mV demonstrating its stability.

Particle size analysis: The average particle size of the Hg-ZnO NPs using DLS method was found to be about 129.5 nm and 126.5 nm for Hg-CuO NPs, both biosynthesized samples exhibited the average particle size fairly higher than the X-ray diffraction method, which might be due to smaller nanoparticles agglomeration.

IN VITRO **ANTIOXIDANT ACTIVITY OF METAL OXIDE NANOPARTICLES AND AQUEOUS HG EXTRACT**

DPPH assay: HN extract showed significantly greater DPPH scavenging action at lower concentrations (12.5 to 50 µg/mL) compared to Hg-ZnO NPs, Hg-CuO NPs and ascorbic acid. However, Hg-ZnO NPs showed higher scavenging activity then HG extract as the concentration was increased. All extracts showed dose dependent inhibition as the concentration was increased.

FRAP assay: HG extract and Hg-CuO NPs showed significantly greater FRAP action at lower concentrations (12.5 to 50 µg/mL) compared to Hg-ZnO NPs, and ascorbic acid. However, Hg-ZnO NPs showed higher scavenging activity as the concentration was increased. All extracts showed dose dependent inhibition as the concentration was increased.

PM assay: Hg-CuO NPs showed significantly greater scavenging action at lower concentrations (12.5 to 50 µg/mL) compared to HG extract, Hg-ZnO NPs, and ascorbic acid. All extracts showed dose dependent inhibition as the concentration was increased.

IN VITRO ANTICANCER ACTIVITY OF METAL OXIDE NANOPARTICLES AND AQUEOUS HN EXTRACT

Cytotoxicity studies of Hg-ZnO NPs, Hg-CuO NPs and HG extract using MTT assay

MTT assay (HEK293 cell line): At lower concentration (6.25 µg/mL), 90% viable cells were present in HG extract while nearly 80% cells were unharmed in Hg- CuO NPs extract. Whereas, in Hg-ZnO NP extract, 60% viable cells can be seen. However, as the concentration was increased (200 µg/mL), less than 50% living cells can be seen in metal oxide nanoparticles and almost 70% unharmed cells were present in HG extract.

MTT assay (A498 cell line): At lower concentration (6.25 µg/mL), metal oxide nanoparticles inhibited more than 70% cancer cells whereas HG extract inhibited very less amount of cancer cells. As the concentration was increased (200 µg/mL), metal oxide nanoparticles killed almost 90% of cancer cells in 48 hrs. These results suggest that the effect of HG extract, Hg- ZnO NP and Hg-CuO NP extract are dose dependent. Metal oxide nanoparticles are ideal choice at 6.25 µg/mL because no significant effect was observed in normal renal cells at this concentration. Lower the IC_{50} value, higher the anticancer activity. SI values > 2 was considered as high selectivity. Cisplatin was used as the standard drug.

Cytotoxicity studies of Hg-ZnO NPs, Hg-CuO NPs and HG extract

XTT assay (HEK293 cell line): At lower concentration (6.25 µg/mL), 90% viable cells were present in HG extract while nearly 80% cells were unharmed in and Hg-ZnO NP and Hg-CuO NPs. However, as the concentration was increased (200 µg/mL), more than 50% living cells can be seen in metal oxide nanoparticles and almost 60% unharmed cells were present in HG extract.

XTT assay (A498 cell line): At lower concentration (6.25 µg/mL), Hg-CuO NPs inhibited more than 60% cancer cells while Hg-ZnO NPs inhibited 50% cancer cells whereas HG extract inhibited 40% of cancer cells. As the concentration was increased (200 µg/mL), metal oxide nanoparticles killed almost 90% of cancer cells in 48 hrs respectively. These results suggest that the effect of HG extract, Hg-ZnO NPs and Hg-CuO NPs extract are dose dependent. Metal oxide nanoparticles are ideal choice at 6.25 µg/mL because no significant effect was observed in normal renal cells at this concentration. Lower the IC_{50}

value, higher the anticancer activity. SI values > 2 was considered as high selectivity. Cisplatin was used as the standard drug.

Apoptosis activity of metal oxide nanoparticles and aqueous Hg extract

DAPI: DAPI staining revealed that all treated A498 cells visibly appeared to exhibit small nuclei with bright chromatin condensation, blebbing, nuclear fragmentation, apoptotic body formation, chromatin condensation, nuclear shrinkage and nuclear blebbing. The control cells, however, remained intact and evenly shaped. The DAPI staining noticeably showed apoptotic morphological changes in HG extract, Hg-ZnO NPs, and Hg-CuO NPs in terms of both nuclear condensation and cell structure loss.

Annexin V-FITC/PI: The percentage of viable, early apoptotic, late apoptotic, and necrotic cells of negative control (untreated), positive control (Cisplatin), HG extract, Hg-ZnO NPs, Hg-CuO NPs and A498 cells treated for 12 hrs, was determined by a flow cytometer. There was almost 100% of negative control A498 culture cells were viable, and no apoptosis occurred after 12 hrs, of incubation. However, treated cells with HG extract, Hg-ZnO NPs and Hg-CuO NPs, the viable cell percentage reduced significantly at IC_{25}, IC_{50} and IC_{75} respectively. Furthermore, the percentage of cells at apoptotic stage increased significantly at all concentrations compared to control group.

The thesis deals with each of the above topics in detail.

CHAPTER VII - SUMMARY AND CONCLUSION

This thesis deals mainly with the preliminary qualitative, quantitative, physicochemical parameters, and GCMS analysis of various crude extracts of *H. nigra, H. ferrugenia, and H. grahamii* leaves. Furthermore, optimization, green synthesis, and characterization of zinc oxide and copper oxide nanoparticles from aqueous leaves extracts of *H. nigra, H. ferrugenia, and H. grahamii* and evaluation of their antioxidant as well as anticancer properties in kidney cancer cell line have been described in different chapters and the summary and conclusion for each chapter have been described below:

Chapter I

This chapter initially deals with the introductory details of nanotechnology and their applications, cancer and its prevalence, The need for choosing the medicinal plant and their advantages were explained in detail. Furthermore, the general information of ZnO and CuO NPs and their applications are given in details.

Chapter II

This section gives the literature relevant to the present study. A summary on the different types of plants used in the biosynthesis of ZnO and CuO NPs has also been given. The literature search provides the background and guidance for the scope of present investigation.

Chapter III

This chapter briefly covers the materials required and instruments used for the study. Initially, chapter deals with collection of plants, preparation of extracts, preliminary physicochemical and phytochemical screening, GCMS analysis, optimization and synthesis of nanoparticles (ZnO NPs and CuO NPs). Furthermore, the instrumental techniques for characterization of synthesized nanoparticles using the techniques like UV-vis spec, FT-IR, XRD, SEM, EDAX, HR-TEM, SAED pattern, particle size distribution, and DLS of ZnO NPs and CuO NPs have been given. At the end, the chapter deals with the assay procedures of antioxidant and anticancer activities.

Chapter IV

This chapter deals with results and discussion of the entire study. Starting from preliminary physicochemical and phytochemical analysis till apoptosis. Briefly, the study revealed that physicochemical analysis of HN showed the presence of trace elements. Preliminary phytochemical analysis revealed the presence of tannins, alkaloids, flavonoids, phenols,

saponins and steroids in various crude extracts of HN and by GC-MS analysis the bioactive compounds were identified of different solvent extracts of HN. The free radical scavenging activity of all solvent extracts of HN leaves exhibited better antioxidant properties.

Furthermore, optimization of nanoparticles using aqueous leaf extract of HN played a key role in synthesizing smaller nanoparticles. Characterization techniques confirmed the shape, size and morphology of synthesized nanoparticles. Biological application such as antioxidant and anticancer activity of Hn-ZnO and Hn-CuO NPs comparing with the plant extract (HN extract) showed dose dependent activity. Green synthesized ZnO and CuO NPs showed significant anticancer activity on A498 cancer cell line.

Metal oxide nanoparticles and HN extract showed significant cytotoxic effects against A498 cell line without killing normal cell line. The evaluation of apoptosis by DAPI confirmed the morphological changes in both the plant extract and metal oxide nanoparticles. Finally, flow cytometry applied to quantitatively detect apoptosis rates through Annexin V-FITC/PI suggested that ZnO, CuO NPs and plant extract induced the early apoptosis rather than necrotic pathway.

Chapter V

This chapter deals with results and discussion of the entire study. Starting from preliminary physicochemical and phytochemical analysis till apoptosis. Briefly, the study revealed that physicochemical analysis of HF showed the presence of trace elements. Preliminary phytochemical analysis revealed the presence of tannins, alkaloids, flavonoids, phenols, saponins and steroids in various crude extracts of HF and by GC-MS analysis the bioactive compounds were identified of different solvent extracts of HF. The free radical scavenging activity of all solvent extracts exhibited better antioxidant properties.

Furthermore, optimization of nanoparticles using aqueous extract of HF played a key role in synthesizing smaller nanoparticles. Characterization techniques confirmed the shape, size and morphology of synthesized nanoparticles. Biological application such as antioxidant and anticancer activity of ZnO and CuO NPs comparing with the plant extract (HF extract) showed dose dependent activity. Green synthesized ZnO and CuO NPs showed significant anticancer activity on A498 cancer cell line.

Metal oxide nanoparticles and HF extract showed significant cytotoxic effects against A498 cell line without killing normal cell line. The evaluation of apoptosis by DAPI confirmed the morphological changes in both the plant extract and metal oxide nanoparticles. Finally, flow cytometry applied to quantitatively detect apoptosis rates

through Annexin V-FITC/PI suggested that ZnO, CuO NPs and plant extract induced the early apoptosis rather than necrotic pathway.

Chapter VI

This chapter deals with results and discussion of the entire study. Starting from preliminary physicochemical and phytochemical analysis till apoptosis. Briefly, the study revealed that physicochemical analysis of HG showed the presence of trace elements. Preliminary phytochemical analysis revealed the presence of tannins, alkaloids, flavonoids, phenols, saponins and steroids in various crude extracts of HG and by GC-MS analysis the bioactive compounds were identified of different solvent extracts of HG. The free radical scavenging activity of all solvent extracts exhibited better antioxidant properties.

Furthermore, optimization of nanoparticles using aqueous extract of HG played a key role in synthesizing smaller nanoparticles. Characterization techniques confirmed the shape, size and morphology of synthesized nanoparticles. Biological application such as antioxidant and anticancer activity of ZnO and CuO NPs comparing with the plant extract (HG extract) showed dose dependent activity. Green synthesized ZnO and CuO NPs showed significant anticancer activity on A498 cancer cell line.

The evaluation of apoptosis by DAPI confirmed the morphological changes in both the plant extract and metal oxide nanoparticles. Finally, flow cytometry applied to quantitatively detect apoptosis rates through Annexin V-FITC/PI suggested that ZnO, CuO NPs and plant extracts induced the early apoptosis rather than necrotic pathway.

CONCLUSION

In conclusion, despite being a toxic plant, Holigarna species possesses many secondary metabolites and radical scavengers, especially polyphenols which acted as capping and stabilizing agents for nanoparticle synthesis. A simple, eco-friendly, and rapid approach for the synthesis of CuO and ZnO nanoparticles accomplished by using HN, HF, and HG leaf extracts showed good radical scavenging activity. Optimization techniques play a key role in nanoparticle synthesis and metal oxide nanoparticles showed significant anticancer activity compared to plant extracts and standard drug. Especially, CuO nanoparticles showed better anticancer activity in all plant extracts compared to ZnO NPs and plant extracts. These results highlight the use of conventional medicinal plants by hybridizing

the advanced nanotechnology for the treatment of cancer which is the primary threat to modern human society.

REFERENCES

1. ISO T. 27687. Nanotechnologies–Terminology and definitions for nano-objects–nanoparticle, nanofibre and nanoplate. 2008.
2. Taniguchi N. On the basic concept of nanotechnology. Proceeding of the ICPE. 1974.
3. Albrecht MA, Evans CW, Raston CL. Green chemistry and the health implications of nanoparticles. Green chemistry. 2006;8(5):417-32.
4. Schodek DL, Ferreira P, Ashby MF. Nanomaterials, nanotechnologies and design: an introduction for engineers and architects. Butterworth-Heinemann; 2009 Mar 24.
5. Ju-Nam Y, Lead JR. Manufactured nanoparticles: an overview of their chemistry, interactions and potential environmental implications. Science of the total environment. 2008 Aug 1;400(1-3):396-414.
6. Klingshirn C. ZnO: material, physics and applications. ChemPhysChem. 2007 Apr 23;8(6):782-803.
7. Wang S, Huang X, He Y, Huang H, Wu Y, Hou L, Liu X, Yang T, Zou J, Huang B. Synthesis, growth mechanism and thermal stability of copper nanoparticles encapsulated by multi-layer graphene. Carbon. 2012 May 1;50(6):2119-25.
8. Sastry AB, Karthik Aamanchi RB, Prasad SR, Murty BS. Large-scale green synthesis of Cu nanoparticles. Environmental chemistry letters. 2013 Jun;11(2):183-7.
9. Chandra S, Kumar A, Tomar PK. Synthesis and characterization of copper nanoparticles by reducing agent. Journal of Saudi Chemical Society. 2014 Apr 1;18(2):149-53.
10. Dhineshbabu NR, Rajendran V, Nithyavathy N, Vetumperumal R. Study of structural and optical properties of cupric oxide nanoparticles. Applied Nanoscience. 2016 Aug;6(6):933-9.
11. Selim YA, Azb MA, Ragab I, HM Abd El-Azim M. Green synthesis of zinc oxide nanoparticles using aqueous extract of Deverra tortuosa and their cytotoxic activities. Scientific reports. 2020 Feb 26;10(1):1-9.
12. Umamaheswari A, Prabu SL, John SA, Puratchikody A. Green synthesis of zinc oxide nanoparticles using leaf extracts of Raphanus sativus var. Longipinnatus and evaluation of their anticancer property in A549 cell lines. Biotechnology Reports. 2021 Mar 1;29:e00595.
13. Njugi W. A constructive approach on lethal plants for medicinal use. Journal of Medical Toxicology and Clinical Forensic Medicine. 2018;4(1):2.
14. Pearson RS, Brown HP. Commercial timbers of India: their distribution, supplies, anatomical structure, physical and mechanical properties and uses. Volumes I & II. Commercial timbers of India: their distribution, supplies, anatomical structure, physical and mechanical properties and uses. Volumes I & II.. 1932.

15. Shukla A, Mehrotra RC. Holigarna (Anacardiaceae) from the early Eocene of Western India and its palaeogeographical and palaeoclimatological significance. Journal of the Geological Society of India. 2016 May;87(5):520-4.
16. Kalase VL, Jadhav VD. Pharmacognostical evaluation of leaf of Holigarna grahamii (wight) kurz: an Endemic plant to western ghats. Asian J Pharm Clin Res. 2013;6(2):105-8.
17. Iyengar MA, Bhat KG, Nayak GK, Rajgopal PK, Singh R. Survey of Medicinal flora of South Kanara-III. Indian Drugs. 1987;25:45-51.
18. Ravi A, Saj OP. Antioxidant and cytotoxic potential of the plant Holigarna arnottiana hook. F. Bark ethanolic extract. World Journal of Pharmaceutical Research. 2015 Mar 27;4(6):1234-44.
19. Nayak SG, Kulkarni SB, Iyengar MA. Histology of the bark and leaf of a toxic plant Holigarna ferruginea march. Ancient Science of Life. 1993 Jan;12(3-4):327.
20. Manilal A, Idhayadhulla A. Potential in vitro antimicrobial efficacy of Holigarna arnottiana (Hook F). Asian Pacific Journal of Tropical Biomedicine. 2014 Jan 1;4(1):25-9.
21. Ahmad R, Ahmad N, Naqvi AA, Shehzad A, Al-Ghamdi MS. Role of traditional Islamic and Arabic plants in cancer therapy. Journal of traditional and complementary medicine. 2017 Apr 1;7(2):195-204.
22. Sridharan M, Kamaraj P, Arockiaselvi J, Pushpamalini T, Vivekanand PA, Kumar SH. Synthesis, characterization and evaluation of biosynthesized Cerium oxide nanoparticle for its anticancer activity on breast cancer cell (MCF 7). Materials Today: Proceedings. 2021 Jan 1;36:914-9.
23. Rutz J, Maxeiner S, Juengel E, Bernd A, Kippenberger S, Zöller N, Chun FK, Blaheta RA. Growth and proliferation of renal cell carcinoma cells is blocked by low curcumin concentrations combined with visible light irradiation. International journal of molecular sciences. 2019 Jan;20(6):1464.
24. Swiatek M, Jancewicz I, Kluebsoongnoen J, Zub R, Maassen A, Kubala S, Udomkit A, Siedlecki JA, Sarnowski TJ, Sarnowska E. Various forms of HIF-1α protein characterize the clear cell renal cell carcinoma cell lines. IUBMB life. 2020 Jun;72(6):1220-32.
25. Wang X, Liu J, Xie Z, Rao J, Xu G, Huang K, Li W, Yin Z. Chlorogenic acid inhibits proliferation and induces apoptosis in A498 human kidney cancer cells via inactivating PI3K/Akt/mTOR signalling pathway. Journal of Pharmacy and Pharmacology. 2019 Jul;71(7):1100-9.
26. Liu R, Pei Q, Shou T, Zhang W, Hu J, Li W. Apoptotic effect of green synthesized gold nanoparticles from Curcuma wenyujin extract against human renal cell carcinoma A498 cells. International Journal of Nanomedicine. 2019;14:4091.
27. Arambewela LS, Arawwawala LD. Standardization of Alpinia calcarata Roscoe rhizomes. Pharmacognosy Research. 2010 Sep;2(5):285.
28. Adeyeye EI, Agesin OO. Dehulling the African yam bean (Sphenostylis stenocarpa Hochst. ex A. Rich) seeds: Any nutritional importance? Note I. Bangladesh Journal of Scientific and Industrial Research. 2007;42(2):163-74.

29. AOAC. Official Method of Analysis of Association of Analytical Chemists. 17th Edn., Association of Official Analytical Chemists (AOAC) International, USA. 2000.
30. Odebiyi OO, Sofowora EA. Phytochemical screening of Nigerian medicinal plants II. Lloydia. 1978 May 1;41(3):234-46.
31. Sunderman Jr FW. Atomic absorption spectrometry of trace metals in clinical pathology. Human Pathology. 1973 Dec 1;4(4):549-82.
32. Herborne JB. Phytochemical methods. A guide to modern techniques of plant analysis. 1973;2:5-11.
33. Evans WC. Trease and Evans' pharmacognosy. Elsevier Health Sciences; 2009 May 27.
34. Roopashree TS, Dang R, Rani SR, Narendra C. Antibacterial activity of antipsoriatic herbs: Cassia tora, Momordica charantia and Calendula officinalis. International Journal of Applied research in Natural products. 2008 Sep 15;1(3):20-8.
35. Noreen H, Semmar N, Farman M, McCullagh JS. Measurement of total phenolic content and antioxidant activity of aerial parts of medicinal plant Coronopus didymus. Asian Pacific journal of tropical medicine. 2017 Aug 1;10(8):792-801.
36. Iqbal E, Salim KA, Lim LB. Phytochemical screening, total phenolics and antioxidant activities of bark and leaf extracts of Goniothalamus velutinus (Airy Shaw) from Brunei Darussalam. Journal of King Saud University-Science. 2015 Jul 1;27(3):224-32.
37. Nahapetian A, Bassiri A. Changes in concentrations and interrelations of phytate, phosphorus, magnesium, calcium, and zinc in wheat during maturation. Journal of Agricultural and Food Chemistry. 1975 Nov;23(6):1179-82.
38. Prabhadevi V, Sahaya SS, Johnson M, Venkatramani B, Janakiraman N. Phytochemical studies on Allamanda cathartica L. using GC–MS. Asian Pacific Journal of Tropical Biomedicine. 2012 Feb 1;2(2):S550-4.
39. Singh D, Rathod V, Ninganagouda S, Hiremath J, Singh AK, Mathew J. Optimization and characterization of silver nanoparticle by endophytic fungi Penicillium sp. isolated from Curcuma longa (turmeric) and application studies against MDR E. coli and S. aureus. Bioinorganic chemistry and applications. 2014 Oct;2014.
40. Zare E, Pourseyedi S, Khatami M, Darezereshki E. Simple biosynthesis of zinc oxide nanoparticles using nature's source, and it's in vitro bio-activity. Journal of Molecular Structure. 2017 Oct 15;1146:96-103.
41. Nm JO, Monika S, Alok S, Alok M, Shruti R. In vitro and in vivo models for antioxidant activity evaluation: A review. Int J Pharm Sci Res. 2010;1(1):1-1.
42. Brand-Williams W, Cuvelier ME, Berset CL. Use of a free radical method to evaluate antioxidant activity. LWT-Food science and Technology. 1995 Jan 1;28(1):25-30.
43. Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Analytical biochemistry. 1996 Jul 15;239(1):70-6.

44. Prieto P, Pineda M, Aguilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. Analytical biochemistry. 1999 May 1;269(2):337-41.
45. Sudha KG, Ali S, Karunakaran G, Kowsalya M, Kolesnikov E, Rajeshkumar MP. Eco-friendly synthesis of ZnO nanorods using Cycas pschannae plant extract with excellent photocatalytic, antioxidant, and anticancer nanomedicine for lung cancer treatment. Applied Organometallic Chemistry. 2020 Apr;34(4):e5511.
46. Todorova J, Lazarov LI, Petrova M, Tzintzarov A, Ugrinova I. The antitumor activity of cannabidiol on lung cancer cell lines A549 and H1299: the role of apoptosis. Biotechnology & Biotechnological Equipment. 2021 Jan 1;35(1):873-9.
47. Boroumand Moghaddam A, Moniri M, Azizi S, Abdul Rahim R, Bin Ariff A, Navaderi M, Mohamad R. Eco-friendly formulated zinc oxide nanoparticles: induction of cell cycle arrest and apoptosis in the MCF-7 cancer cell line. Genes. 2017 Oct;8(10):281.
48. Murali M, Mahendra C, Rajashekar N, Sudarshana MS, Raveesha KA, Amruthesh KN. Antibacterial and antioxidant properties of biosynthesized zinc oxide nanoparticles from Ceropegia candelabrum L.–an endemic species. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy. 2017 May 15;179:104-9.

List of Publications

1. **Vinayak Lokapur,** Varsha Jayakar, Manjula Shantaram*. "Preliminary phytochemical screening, physicochemical analysis and in *in-vitro* antioxidant activity of selected Holigarna species- Endemic plant species of Western Ghats". Biomedicine, 2020; 40(4): 460-466. ISSN: 0970 2067.
2. **Vinayak Lokapur,** Varsha Jayakar, Manjula Shantaram*. "Phytochemical Investigation, Chemical composition and *In-vitro* antioxidant activities of various crude extracts of *Holigarna ferrugenia* Marchand". **Accepted** in Medicinal Plants Journal.
3. **Vinayak Lokapur,** Varsha Jayakar, Divakar M. S., Raju Krishna Chalannavar, Lyned Lasrado, Manjula Shantaram. "ZnO Nanoparticles with spectroscopically controlled morphology, bioinspired from unexlored plant *Holigarna grahamii* (Wight) Kurz and delving its antioxidant as well as cytotoxic activities on A498 cell line". **Under review** in Materials Today Communication.

Award paper - Dr. Yellapragada Subbarao Memorial Award

Vinayak Lokapur, Varsha Jayakar, Manjula Shantaram. **Preliminary phytochemical screening, physicochemical analysis and *in-vitro* antioxidant activity of selected Holigarna species- Endemic plant species of Western Ghats.** 42nd International Conference of IABMS on **'Recent Trends and Advances in Biomedical Sciences – An Integrated Approach'** organised by Nitte (Deemed to be University), Mangalore on 25th – 27th November, 2021.

Poster presentation

"Physicochemical evaluation, preliminary phytochemical investigation and *in-vitro* antioxidant activity of selected endemic plants of Western Ghats" during the 40th Annual Conference of Indian Association of Biomedical Scientists (IABMS) on "Biomedical research and innovation to enhance Quality of life" held from 31st Oct – 2nd November 2019 at Chettinad Hospital and Research Institute (CHRI) Kelambakkam.

Workshop and Conferences Attended

1. Participated in the DBT Ministry of Science and Technology, New Delhi Sponsored Ten Days National workshop on "Plant Microbial Interaction for the production of the Therapeutic Compounds" held from 17th – 26th October 2019 at Kuvempu University.
2. Participated in the Dr. Sarada Subramaniam Preconference workshop on Handling of laboratory animals & Zebra fish in biological research" in the 40th Annual Conference of Indian Association of Biomedical Scientists (IABMS) on "Biomedical research and innovation to enhance Quality of life" held from 31st Oct 2019 at Chettinad Hospital and Research Institute (CHRI) Kelambakkam.
3. Participated in the Interactive Workshop on "Arecanut and Human Health" held during 24th -25th July 2018 at Central Plantation Crops Research Institute, Kasaragod, Kerala.
4. Participated in Two Day Lecture Series in Molecular Genetics held on 24th and 25th August 2018 at K S Hegde Medical Academy, Deralakatte, Mangaluru in association with Karnataka Science and Technology Academy (KSTA).
5. Participated in workshop on "Basics of cell culture and application in drug discovery" during the International Conference on "Current concepts on the role of Indian medicine and phytoceuticals in maintenance of health" under the auspices of 39th Annual Summit of Indian Association of Biomedical Scientists (IABMS) held from 15-17th November 2018 at Jnana kaveri Post Graduate Centre of Mangalore University Chikka Aluvara, Kodagu.
6. Participated in workshop on "DNA Damage Studies" at International Conference on Radiation Biology under the auspices of Indian Society of Radiation Biology theme being "Advances in Radiation Biology-Radiotherapy and Radiation Countermeasures" held on 4th October 2018 at K S Hegde Medical Academy, Deralakatte, Mangaluru.